

## MTA1 induced angiogenesis, migration and tumor growth is inhibited by *Glycyrrhiza glabra*

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**Abstract**—Angiogenesis is an important host process that interacts with cancer cells to promote growth, invasion and metastasis. Vascular endothelial growth factor (VEGF) and Metastasis Associated protein (MTA1) are known to play a major role in angiogenesis. The recombinant VEGF and MTA1 proteins were used to induce proliferation and cell migration in MDA-MB-231 cells. Here we investigated the antiangiogenic and antitumor activity of *G. glabra* F6 (G1) on VEGF and MTA1 induced angiogenesis. The angio inhibitory activity of *G. glabra* F6 (G1) was confirmed by its inhibition of angiogenesis in in vivo assays, peritoneal and chorioallantoic membrane assay. Reduction in the levels of the cytokine VEGF and microvessel density count in the peritoneum of mice treated with *G. glabra* F6 (G1) indicated that the plant extract decreased VEGF production. It also inhibits the neovascularization in CAM induced by VEGF and MTA1. These findings not only suggest a potential role of VEGF or MTA1 in tumour angiogenesis but it is also an effective beginning to explore mechanism of metastasis and cancer therapy strategy targeting MTA1. Our results suggest that the extract from the roots of *G. glabra* may be a potential supplemental source for cancer therapy.

**Keywords**—Angiogenesis, Inhibition, Vascular endothelia growth factor (VEGF), Metastasis Associated protein (MTA1), *Glycyrrhiza glabra*

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### I. INTRODUCTION

Angiogenesis, the growth of new vessels from pre-existing vasculature, is a critical step in tumor progression [1]. New blood vessels are required to support the growth of a tumor beyond the size of about 1–2 mm<sup>3</sup>, to supply oxygen and nutrients to proliferating tumor cells and for metastasis formation [2-3]. Research in angiogenesis inhibition as a therapeutic strategy against cancer started around 1971, when Folkman postulated that tumor growth is dependent on angiogenesis [4]. In the past two decades, inhibitors of angiogenesis have been developed for clinical use [5]. Most notable angiogenesis inhibitors target the vascular endothelial growth factor (VEGF) signaling pathway, such as the monoclonal antibody bevacizumab (Avastin, Genentech/Roche) and two kinase inhibitors sunitinib (SU11248, Sutent, Pfizer) and sorafenib (BAY43-9006, Nexavar, Bayer). In cancer, there is a balance of pro- and anti-angiogenic factors. However when this balance is disturbed, it results in the so-called ‘angiogenic switch’ [6]. Tumor cells secrete a number of pro-angiogenic factors that stimulate the proliferation and migration of endothelial cells, resulting in the outgrowth of new capillaries into the tumor [7]. Angiogenesis is regulated by many cytokines including proangiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), placental growth factor (PIGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), platelet-derived growth factor (PDGF), angiopoietin, angiogenin and interleukin (IL-8) [8-10]. While approximately, 60% of human cancer expresses VEGF, avastin that inhibits the expression of VEGF or its receptor is found to be effective especially when used in combination with chemotherapy. The VEGF gene is regulated by hypoxia and is under the control of transcription factor Hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) [11]. HIF-1 $\alpha$  is over expressed in premalignant lesions in the colon, colorectal cancer and its metastasis and is also considered to be an independent indicator of poor prognosis [12].

Metastasis Associated Protein (MTA1), the founding member of the MTA family of genes, and is up-regulated in human tumors. MTA1 and MTA2 are integral subunits of the nucleosome remodeling histone deacetylation (NuRD) complex and are implicated in chromatin-modifying role [13]. Several studies have identified various roles for MTA1 in normal mammary gland development and human breast cancer progression, including cell proliferation and invasiveness [14]. A recent report suggested that MTA1 may possibly be involved in the regulation of gene expression by covalent modification of histone proteins playing a role in histone deacetylation and transcriptional control [15]. MTA1 and HIF-1 $\alpha$  proteins are expressed in malignant metastatic tumor cells and are therefore expected to have important roles in tumor progression and metastasis during the development of cancer. The cross-talk between MTA1 and HIF-1 has been investigated. It

also has been shown that MTA1 enhances the stability and transcriptional activity of HIF-1 by recruiting HDAC in human breast cancer cells [13]. According to a previous report that HDAC induces angiogenesis, the deacetylation activity of MTA homologues may potentially be important in regulation of angiogenesis or metastasis.

A balance between angiogenic and antiangiogenic factors has given rise to a significant interest in the use of exogenous antiangiogenic agents for the treatment of tumors and it has been demonstrated that antiangiogenic treatment retards tumor growth. Almost 60% of drugs approved for cancer treatment are of natural origin. Vincristine, etoposide, taxanes and camptothecins are all examples of plant-derived anticancer compounds. *Glycyrrhiza glabra* (Licorice) is a favourable herb used in food and medicinal remedies for thousands of years. This herb has long been valued as a demulcent (soothing, coating agent), to relieve respiratory ailments (such as allergies, bronchitis, cold, sore throats and tuberculosis), stomach burn including heart burn from reflux or any other cause, gastritis, inflammatory disorders, skin diseases and liver problems. Glycyrrhizin, one of the main active ingredients of *G. glabra* is believed to contribute to the herb's many healing properties [16]. Licorice roots contain flavonoids and chalcones [16]. Isoliquiritigin (ISL) and licochalcon-A members of the flavonoids have been investigated to have antioxidant, antitumor, anti inflammatory and antiangiogenic activities [18-21]. ISL and licochalcone have been shown to induce apoptosis in colon, gastric and prostate cancer cells. However the antitumor mechanisms of these compounds have not been well defined. ISL, isoliquiritin, liquiritigenin and isoliquiritin-apioside are licorice-derived flavonoids that have been implicated to inhibit angiogenesis and tumor growth [22].

We in our previous report have shown that crude extract of *G. glabra* (water extracts) inhibits the angiogenic activity [16]. In the present paper attempts have been made to further investigate by activity guided purification, the active principle from *G. glabra* which is responsible for the antiangiogenic activity. Because angiogenesis is a prerequisite for not only the growth of tumor but also for tumor metastasis. We have identified the ethyl acetate and methanol (80:20) extract of *G. glabra* to possess anti proliferation and antiangiogenic activity. We have shown that MTA1 is co-expressed by tumor cells and *G. glabra* extract inhibits angiogenic processes. And also, have shown the MTA1 is secreted along with VEGF in the ascites obtained from mouse mammary carcinoma and also MTA1, is a proangiogenic protein. Inhibition of MTA1 induced tumor growth and angiogenesis proves to be effective in proangiogenic therapy.

## II. MATERIALS AND METHODS

Human Umbilical Vein Endothelial cells (HUVECs) were purchased from Cambrex, USA. MDA-MB-231 was purchased from National Center for Cell Science (NCCS), Pune, INDIA. The cells were cultured in 25 cm<sup>3</sup> tissue culture flask (NUNC, USA) and grown using Endothelial Growth Medium (EGM-2) for endothelial cells and Dulbecco's Minimum Essential Medium (DMEM) with 10% Fetal Bovine Serum (FBS), Streptomycin and Penicillin from GIBCO laboratories, Grand Island, NY, USA. Complete medium was prepared according to the manufacturer's protocol. Incubation was carried out in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C and upon reaching to confluency; the cells were passaged after trypsinization. Mice (6–8 weeks Swiss Albino strain) were obtained from the animal house, Department of Zoology, University of Mysore, Mysore, India. All the experiments were approved by the Institutional animal care and use committee of the University of Mysore, Mysore.

### 2.1 Plant Material

The medicinal plant *Glycyrrhiza glabra* (*Leguminosae/ Fabaceae*) (roots) was collected from Western Ghats of Karnataka, India. The plant was identified by a Taxonomist and the identification was confirmed by depositing the voucher specimens in the Herbarium of Department of Studies in Botany, University of Mysore, Mysore, India, by comparing with available voucher specimens. Solvents petroleum ether, hexane, benzene, chloroform, ethyl acetate, acetone, methanol and chemical silica gel were of highest analytical grade and obtained from Sisco research laboratory, Mumbai, India.

### 2.2 Fractionation and purification of the active compound from *Glycyrrhiza glabra*

Dried and powdered roots of *G. glabra* (1 kg) was subjected to polarity based soxhlet fractionation using solvents such as petroleum ether, hexane, benzene, chloroform, ethyl acetate, acetone, methanol, and ethanol. All the solvent extracts were subjected to evaporation to retain the residue. The methanolic extract was finally purified by Silica gel column chromatography (Merck 70-230 mesh) and eluted with ethyl acetate: methanol (80:20). The collected fraction was subjected to thin layer chromatography (data not shown) using chloroform: ethyl acetate: methanol and were pooled based on the bands (6 bands) that appeared on TLC. After pooling, the obtained fractions were evaporated to dryness and known concentration of each fraction was tested for activity. The compound F6 (G1) showed maximum effect.

## 2.3 *In vivo* angiogenesis assays

### 2.3.1 *In vivo* growth of Ehrlich ascites tumor (EAT) cells and peritoneal angiogenic assay

EAT cells ( $5 \times 10^6$ ) were injected intraperitoneally (i.p) into mice (5 groups of mice, 6 in each group) and growth was recorded everyday from the day of transplantation. To verify the effect of plant extract to inhibit tumor growth and angiogenesis mediated by EAT cells *in vivo*, the F6 (G1) extract (100  $\mu$ l) was injected into the peritoneum of the EAT bearing mice everyday from the 6<sup>th</sup> day of transplantation. The body weight of the mice was monitored from the 1<sup>st</sup> day till the 12<sup>th</sup> day. On the 12<sup>th</sup> day, the animals were sacrificed and the volume of the ascites formed both in untreated and treated mice were recorded. The pelleted cells were counted by trypan blue dye exclusion method using a hemocytometer. The animals were dissected to observe the effect of the extract on peritoneal angiogenesis [16]. All the experiments were approved by the institutional animal care and use committee, University of Mysore, Mysore, India.

### 2.3.2 Immunohistological analysis (H&E staining)

To determine the effect of F6 (G1) extract to inhibit the microvessel density. EAT bearing mice were treated regularly with the F6 (G1) extract from the 6<sup>th</sup> day of transplantation. On the 12<sup>th</sup> day, the animals were sacrificed and the peritoneum from treated or untreated mice was fixed in 10% formalin. Sections (5 $\mu$ m) were made from paraffin embedded peritoneum using automatic microtome (SLEE Cryostat) and stained with Hematoxylin and Eosin. Microvessel counts were done by Hot-Spot method [23] and the images were photographed using Leitz-DIAPLAN microscope with attached CCD camera.

### 2.3.3 Enzyme Linked Immunosorbent Assay (VEGF/MTA1-ELISA)

We have standardized a sensitive and specific quantification indirect ELISA system for MTA1. This assay was performed as previously described [24] with modification. About 100  $\mu$ l of cytosolic extract of EAT cells were coated to 96 well microplate using coating buffer and incubated at 4°C overnight. To generate a standard curve, purified MTA1 was diluted in coating buffer at concentrations ranging from 230 pg/ml to 23  $\mu$ g/ml. The diluted MTA1 protein standards and aliquots of cytosolic extract of EAT (100  $\mu$ l/well) were coated to the 96-well microtiter ELISA plates (Nunc MaxiSorp™, Nunc, USA) using a coating buffer (50 mM sodium carbonate buffer, pH 9.6) at 4°C overnight. Subsequently, blocked for 1 h with blocking buffer (5% BSA/PBS). Anti-MTA1 polyclonal antibody (dilution 1:1000), 100  $\mu$ l/well was added and incubated for 2 h at 37°C followed by incubation with 100  $\mu$ l of secondary antibody (1:5,000) conjugated to alkaline phosphatase. And developed with 100  $\mu$ l of p-nitrophenyl phosphate solution.

### Analysis of VEGF level in EAT Mice treated with F6 (G1)

In brief, 100  $\mu$ l of ascites sample from F6 (G1) extract treated or untreated mice was coated to 96 well microplate using coating buffer and incubated at 4°C overnight. Wells were washed and blocked with blocking buffer (5% skimmed milk powder in PBS) for 2 h at 37°C, followed by incubation with anti VEGF 165 antibodies (1:1000). Recombinant anti mouse VEGF 165 was used to set up the standard curve. After incubation for 2 h, the wells were washed before treating with 100  $\mu$ l/well of goat anti-rabbit IgG conjugated to alkaline phosphatase (1:2000). Incubation was continued for another 2 h at RT and plate was washed prior to addition of 100  $\mu$ l of the substrate p-nitro-phenyl phosphate (pNPP). After incubation for 30 min at RT, the reaction was terminated by adding 0.1 N NaOH and the absorbance at 405nm were measured in a Medispec ELISA reader.

### 2.3.4 Chorioallantoic membrane assay

CAM assay was performed as described previously [25]. In brief, the fertilized eggs were incubated at 37°C in a humidified and sterile atmosphere for 10 days. A window was made under aseptic conditions on the eggshell to check for proper development of the embryo. The window was made on 5<sup>th</sup> day, resealed and allowed to develop further. On the 12<sup>th</sup> day, saline, recombinant VEGF (10 ng/egg), recombinant MTA1 (10 ng/egg) with or without the extract (60  $\mu$ l/egg) were air dried on sterile glass cover slips. The window was reopened and the cover slip was inverted over the CAM. The window was closed again, the eggs were returned to the incubator for another 2 days. The windows were opened on the 14<sup>th</sup> day and inspected for changes in the microvessel density in the area under the coverslip and photographed.

## 2.4 *In vitro* angiogenic assays

### 2.4.1 Tube formation assay

In order to study if *G. glabra* F6 (G1) extract inhibits VEGF or MTA1 induced the formation of capillary like tubes in Human Umbilical Vein Endothelial Cells (HUVECs) a tube formation assay was performed as described in earlier reported by us [26]. Briefly, a 96-well plate was coated with 50  $\mu$ l of Matrigel (Becton Dickinson Labware, Bedford, MA), which was allowed to solidify at 37°C for 1 h. HUVECs ( $5 \times 10^3$  cells per well) were seeded on the Matrigel and cultured in EGM media containing different concentrations of VEGF or MTA1 protein (10 ng/ml) or treated with F6 (G1) (60  $\mu$ g/ml) respectively. The cells were incubated at 37°C and 5% CO<sub>2</sub>, for 16 h. The next day complete tubes from randomly chosen fields were counted and photographed under an Olympus inverted microscope (CKX40; Olympus, New York, NY) connected to a digital camera at 40X magnification.

#### 2.4.2 <sup>3</sup>[H] Thymidine uptake assay

In order to verify for the in vitro effect of *G. glabra* F6 (G1) extract on proliferation of MDA-MB-231 cells induced by MTA1 this assay was performed as described earlier [27-28]. Briefly MDA-MB-231 (1x 10<sup>5</sup> cells per well) were seeded in a six-well plate cultured in DMEM media supplemented with 10% FBS, 1 mg/ml penicillin/streptomycin and grown in 5% CO<sub>2</sub> at 37°C for two days. <sup>3</sup>[H] thymidine (1 µCi/ml of medium) were added prior to addition of VEGF (10 ng/ml) or MTA1 (10 ng/ml) with *G. glabra* F6 (G1) extract (60 µg/ml) at different time intervals (0, 6, 12, 24 & 48 h) respectively. Similarly in other set of experiment, the MDA-MB-231 cells were treated with VEGF (10 ng/ml) or MTA1 (10 ng/ml) with *G. glabra* F6 (G1) extract at different concentrations (0, 10, 20 40 & 60 µg/ml). After 48 h, the cells were trypsinized and processed for liquid scintillation counting.

#### 2.4.3 Wound healing assay

In order to verify for the in vitro effect of *G. glabra* F6 (G1) extract on migration of MDA-MB-231 cells induced by MTA1 the wound healing assay was performed. The assay was performed as described earlier [29]. In brief, MDA-MB-231 (2x 10<sup>5</sup> cells per well) were seeded in a six-well plate in complete medium and incubated overnight at 37°C and 5% CO<sub>2</sub>. The cells were serum starved overnight and a wound was scratched on the monolayer using a sterile pipette tip. The plates were washed with PBS twice to remove any detached cells. The cells were treated with mitomycin C (10 ng/ml) for 2 h prior to addition of growth factors with SFM or VEGF (10 ng/ml) or MTA1 (10 ng/ml) or treated with *G. glabra* F6 (G1) (60 µg/ml) in basal media. The wound was photographed subsequently at 0 h, 12 h and 24 h to visualise the closure of the wound area. The distance moved by cells into the wounded area was enumerated manually.

#### 2.5 *G. glabra* F6 (G1) extract inhibits VEGF and MTA1-induced VEGF gene expression

In order to verify the effect of *G. glabra* F6 (G1) extract on VEGF gene expression in MDA-MB-231 cells induced by MTA1 the transient transfection assay was performed. The assay was performed as described earlier [27, 30]. In brief, MDA-MB-231 (2x 10<sup>5</sup> cells per well) were seeded in six-well plates and cultured at 37°C with 5% CO<sub>2</sub> to 60-70% confluency. On the subsequent day, cells were transfected (calcium phosphate transfection kit, Promega, USA) with 2 µg of VEGF promoter-luciferase reporter constructs containing the 5' flanking region (-2068 bp) of human VEGF gene promoter coupled to promoter-less luciferase gene in vector backbone pcDNA3 and 2 µg of the β-galactosidase expression vector RSV-β-gal as an internal control. The transfected cells were incubated 37°C with 3% CO<sub>2</sub> prior to addition of either VEGF (10 ng/ml) or MTA1 (10 ng/ml) or with *G. glabra* F6 (G1) extract (60 µg/ml). Cells were washed once with PBS and were serum starved for 48 h. Cells were washed once again with PBS and lysed with reporter lysis buffer. Luciferase (Luc) activity of the cell extract was determined using the luciferase assay system as per manufacturers instruction. β-galactosidase (β-Gal) activity was determined by measuring hydrolysis of O-nitrophenyl β-D-galactopyranoside using 50 µL of cell extract at 37°C for 2 h. Absorbance was measured at A<sub>405</sub> and normalized. Luciferase activity was determined using 50 µL of cell extract. The reaction was initiated by injection of 100 µL of luciferase assay substrate. Relative luciferase activity (defined as VEGF reporter activity) was calculated as RLU (relative light units per 50 µL cell extract)/β-Gal activity (A<sub>405</sub> per 50 µL cell extract per 2 h).

### III. STATISTICAL ANALYSIS

Unless stated otherwise, all experiments were performed in triplicates. Wherever appropriate, the data were expressed as the mean ± SD and means were compared using one-way analysis of variance. Statistical significance of differences between control, VEGF and MTA1 treated cells were determined by Duncan's multiple range test (DMRT). For all tests, *P* < 0.05 was considered statistically significant. All of the analyses were performed using the SPSS for Windows, version 13.0 (SPSS Inc.).

### IV. RESULTS

#### *In vivo* antitumor and antiangiogenic potential of *G. glabra* fraction 6 (G1) of methanolic extract

In our previous paper we have shown that from *G. glabra* crude extract inhibits angiogenesis [16]. The results on further purification fractionation of the crude extract of *G. glabra* with activity guided purification of biological active molecule revealed the methanolic extract contained antiangiogenic activity. On further fractionation of the methanolic extract using different ratios of ethyl acetate: methanol, the antiangiogenic activity was traced to fraction F6 of the total 6 fractions. The data shown in fig. 1 clearly indicates that fraction F6 (G1) showed *in vivo* antitumor and antiangiogenic activity along with suppressing of tumor cell growth and proliferation.

#### Suppression of peritoneal angiogenesis by *G. glabra* F6 (G1)

The results shown in (Fig 2A) indicate, when compared to the 100% growth of ehrlich ascites tumor (EAT) in the peritoneum of mice there was nearly 50% decrease in the growth of tumor in the mice treated with F6 (G1) (100 µg/day) for tumor growth period. It is also shown in fig. 2B, C that upon treatment of F6 (G1) the

volume of ascites and total number of cells in the peritoneum of F6 (G1) treated animals significantly decreased when compared to the control animals.

#### **G. glabra F6 (G1) inhibits VEGF production in EAT cells**

We have detected 138ng/ml VEGF and 22 ng/ml of MTA1 in either ascites or cytosolic extract of EAT respectively. The representative fig. 2D indicates that F6 (G1) decreases the secretion of angiogenesis factor VEGF in order to exert its antiangiogenic activity *in vivo*.

H & E immunostaining

Fig. 2E is shown the microvessel density of the mouse peritoneum with and without F6 (G1) treatment, it is evident from the results that MVD has been considerably decreased in the peritoneum of F6 (G1) treated mouse as compared to that of EAT bearing control mice.

#### **VEGF and MTA1 induced angiogenesis in CAM is inhibited by G. glabra F6 (G1)**

In non-tumor context chorioallantoic membrane (CAM) of the chick embryo provides a unique model for investigating the process of new blood vessel formation and vessel responses to antiangiogenic agents. Using this model, we examined the *in vivo* antiangiogenic activity of F6 (G1). Formation of new blood vessels either in presence of VEGF or MTA1 was evident in our results as shown in fig 3A, B respectively. The VEGF or MTA1 induced CAM treated with F6 (G1) showed significant decrease in neo-vascularization (Fig. 3C, D)

#### **G. glabra F6 (G1) inhibits tube formation of HUVECs in vitro**

The HUVECs adhered to the Matrigel surface within 20-24 h and form branching, anastomoses network of capillary like tubules with multicentric junctions. As shown in fig. 4A, B VEGF (10 ng) or MTA1 (10 ng) induced tube formation. The F6 (G1) fraction effectively inhibited tube formation induced by VEGF and MTA1 as is shown in fig. 4C, D respectively.

#### **G. glabra F6 (G1) inhibits VEGF or MTA1 induced proliferation in MDA-MB-231 in vitro**

In order to verify the *in vitro* effect of F6 (G1) on either VEGF or MTA1 facilitated proliferation, a proliferation assay was performed using human breast metastatic cancer MDA-MB-231 cells. As is shown in fig. 5A, there was time dependent increase in the proliferation of MDA-MB-231 cells in presence of VEGF or MTA1. However VEGF or MTA1 induced proliferation was highest at 48 hr when compared to other time periods. Inclusion of F6 (G1) (60 µg/ml) in proliferation assay along with VEGF or MTA1, significantly reduced proliferation of tumor cells. Likewise data in Fig. 5B clearly indicates that F6 (G1) inhibits proliferation in a dose dependent manner.

#### **G. glabra F6 (G1) inhibits VEGF or MTA1 induced cell migration in MDA-MB-231 in vitro**

Wound healing or scratch assay is considered to be an assay to verify the migration of tumor cells and its useful to validate antiangiogenic effects of novel molecule. The data shown in Fig.6A clearly indicate that there is wound closure at 24 hr in presence of VEGF or MTA1. However cell migration induced by either VEGF or MTA1 could be effectively inhibited by F6 (G1) (60 µg/ml). Quantitative analysis of wound healing assay (Fig. 6B) showed that in MTA1 treated of MDA-MB-231 showed more number of cells was involved in closing the wound as either compared to that of SFM or VEGF or *G. glabra* F6 (G1).

#### **G. glabra F6 (G1) inhibits VEGF or MTA1 induced VEGF gene expression in MDA-MB-231**

The effect of *G. glabra* F6 (G1) extract on VEGF or MTA1 induced VEGF gene expression in metastatic breast cancer cell line MDA-MB-231 was studied by using VEGF promoter-luciferase reporter assay. Results showed that, expression of VEGF was substantially increased on treatment of VEGF or MTA1 (10 ng/ml) protein (Fig. 7). Subsequently, combination of *G. glabra* F6 (G1) extract (60 µg/ml) with VEGF or MTA1 (10 ng/ml) reduced the VEGF gene expression in breast cancer MDA-MB-231 cells.

## **V. DISCUSSION**

MTA1 was previously identified as a metastasis-promoting gene over-expressed in both rat and human cancer cell lines. The expression of MTA1 in human metastatic breast cancer cell line MDA-MB-231, was determined to be approximately 4 times higher than in MDA-MB-468, which is a non-metastatic cell line [31]. In this paper we are presenting data indicating that MTA1 is an angiogenic protein. Our result indicates that MTA1 is secreted into ascites in mice bearing EAT cells. Tumor growth and metastasis are dependent on the formation of new blood vessels. The most elegant investigation of the correlation between the onset of angiogenesis and tumor growth was carried out by Folkman *et.al* [32]. Inhibitors of angiogenesis block any of the steps in the angiogenic cascade, including proliferation and attachment of endothelial cells to the extracellular matrix proteins, migration and invasion through the matrix, which is required for the capillary sprouting and morphogenesis in a thin tube meshwork and stabilization. Given that angiogenesis is essential for tumor growth, the antitumor effects of *G. glabra* F6 (G1) may correlate with its antiangiogenic activity. *In vivo* experimental studies have demonstrated that tumor growth and peritoneal angiogenesis have been inhibited by *G. glabra* F6 (G1). Although MTA1 is over expressed in a variety of human metastatic cancer cell lines and cancerous tissues, the role of this protein in particular steps of metastatic process has not yet been clarified [13]. Our investigation also indicates that MTA1 induces migration of MDA-MB-231 cells as effectively as VEGF.

This activity is inhibited by *G. glabra* F6 (G1). This result indicates that both MTA1 and VEGF may adopt the same signalling pathways that control the migration of cells in cancer. *G. glabra* F6 (G1) inhibits the growth of tumor both *in vitro* and *in vivo* as is shown in our data on inhibition of proliferation of MDA-MB-231 cells and the growth of EAT cells *in vivo*. In support of this, Nawa *et.al* [33] has shown that the concentration of MTA1 is associated with the rate of proliferation of metastatic and non-metastatic cell lines, where cells expressing more MTA1 are also more metastatic. There are several reports in literature indicating that plants contain potential antiangiogenic active molecules. Deepak *et.al* has shown that a glycoprotein from *Urginea indica* inhibits angiogenesis which is mediated by NF-kB [34]. Our result on VEGF gene expression studies indicates that MTA1 *per se* regulates the expression of VEGF. Our data also reveals that in MDA-MB-231 cells there is an autocrine regulation of VEGF gene expression by VEGF. These results suggest that MTA1 acts synergistically with VEGF to regulate angiogenesis during metastasis. Further, inhibition of either MTA1 or VEGF induced expression of VEGF by *G. Glabra* F6 (G1) indicates that the plant extract also act at transcriptional level in order to reveal its efficacy as antiangiogenic molecule. Gururaj *et.al* have shown that curcumin inhibits VEGF gene expression via, NF-kB [35]. Our previous studies on crude extracts from various plants like *Glycyrrhiza glabra*, *Anacardium occidentale* L, *Terminelia bellirica*, *Tinospora cordifolia* and *Dioscoria bulbifera* L have shown to inhibit growth of EAT cells in mice [16, 27, 36-38]. Given that angiogenesis is essential for tumor growth, the antitumor effects of *G. glabra* F6 (G1) may correlate with its antiangiogenic activity. The current results have also shown that there is inhibition of neovascularization by *G. glabra* F6 (G1) extract induced either by VEGF or MTA1 protein in the CAM, suggesting that *G. glabra* F6 (G1) can directly inhibit neovascularisation in a non-tumor context. Inhibition of fluid accumulation, tumor growth, and microvessel density by neutralization of VEGF by *G. glabra* F6 (G1) demonstrates the underlining importance of VEGF and MTA1 in malignant ascites formation. Since there is inhibition of neovascularization by *G. glabra* F6 (G1), this supports the view that *G. glabra* F6 (G1) may repress the expression of VEGF-like factors or inhibit the secretion of such factors, thereby inhibiting the accumulation of ascites fluid and formation of new blood vessels. Further evidence for the antiangiogenic potential of *G. glabra* F6 (G1) comes from the current results on inhibition of the extent of proliferating endothelial cells in the peritoneal lining of tumor-bearing mice. A significant decrease in peritoneal angiogenesis on peritoneal wall confirmed the antiangiogenic activity of *G. glabra* F6 (G1). Research has demonstrated that the density of microvessels was almost doubled in tumors from patients with metastasis. Thus, an antiangiogenic agent could conceivably block the paracrine action of tumor cells and hence suppress the proliferation and survival of tumor cells. Inhibition of VEGF gene expression by *G. glabra* F6 (G1) should also be reflected by the levels of VEGF in the ascites secreted by the EAT cells. The current results on quantification of the cytokine in the ascites of EAT bearing mice have clearly indicated that *G. glabra* F6 (G1) efficiently decreases the level of VEGF in an *in vivo* model system. A decrease in ascites formation *in vivo* and in VEGF levels in ascites bears significant importance in terms of a clinical correlation with inhibited ascites formation in human tumors.

In conclusion, our results suggest that the extract from *G. glabra* F6 (G1) may be a potential supplemental source for cancer treatment; this study showed that the extracts could efficiently reduce the rate of VEGF and MTA1 induced proliferation and wound healing, suggest that combinational therapy can be used as in treating human breast cancer. Understanding the molecular mechanism of *G. glabra* F6 (G1) contributes to development of new strategies to inhibit tumor migration and metastasis.

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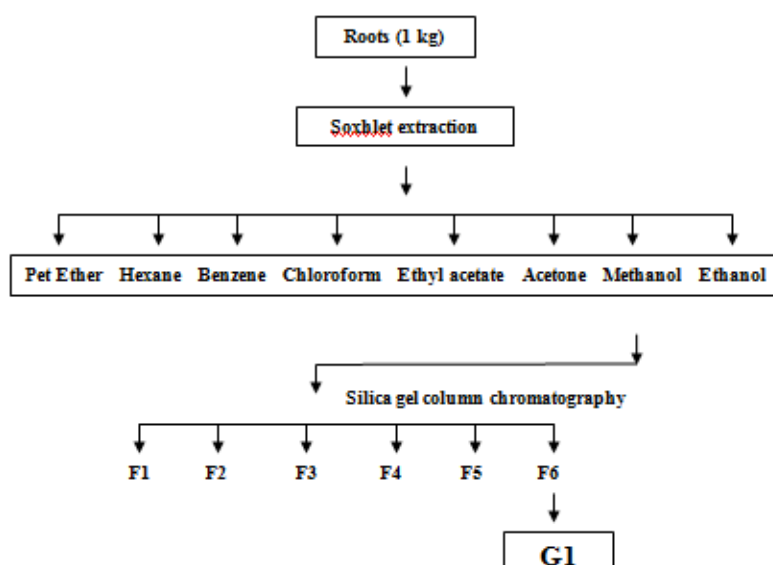
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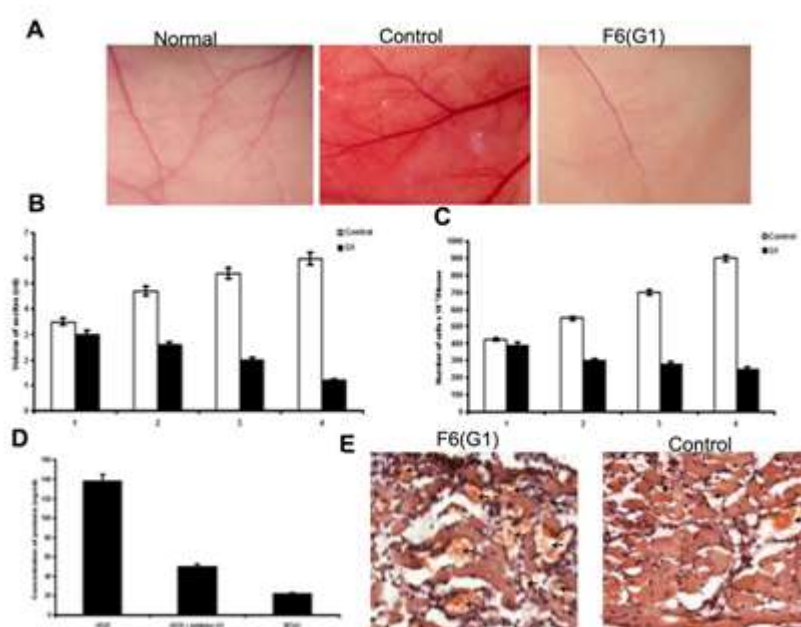
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## Figures and Legends



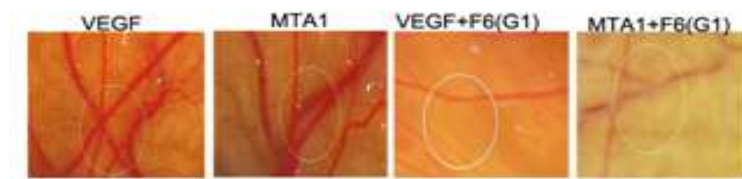
**Fig.1** The methanolic extract obtained by Soxhlet extraction was further fractionated using a silica gel column chromatography and various ratios of solvents. The anti-angiogenic activity was seen in the fraction eluted with ethyl acetate: methanol (80:20). Further, six fractions were obtained of which F6 (G1) showed substantial anti-angiogenic effect.



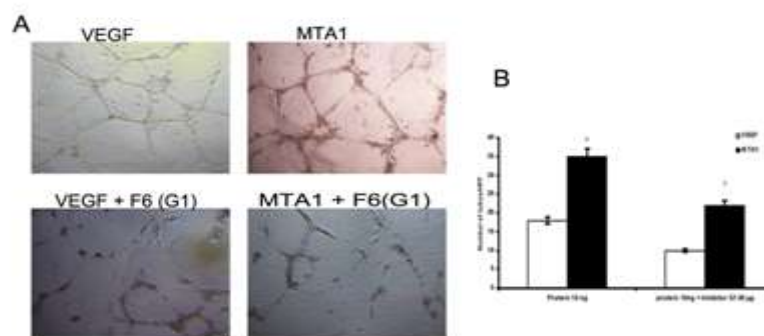
**Fig. 2A** EAT bearing mice treated with or without F6 (G1) (100 µg/dose) were sacrificed on the 12<sup>th</sup> day. The animals were dissected and observed for extent of neovascularization. Inhibition of neovascularization is evident in F6 (G1) treated mice. **B, C** The mice treated with or without F6 (G1) were sacrificed after each dose and the volume of ascites formed was noted. Cells were collected and cell number was counted by trypan blue exclusion method. A drastic decrease in the ascites volume and cell number was observed in a dose dependent manner in the mice treated with F6 (G1). **D** ELISA was performed with the ascites and cytosolic extract from EAT. VEGF and MTA1 were expressed notably in a higher concentration and it was decreased on treatment with F6 (G1). **E**



The peritoneum of control and as well as F6 (G1) treated EAT bearing mice was embedded in paraffin and 5µm sections were taken using microtome. The sections were stained with Hematoxylin and eosin and observed for microvessel density. Arrows indicate the microvessels.

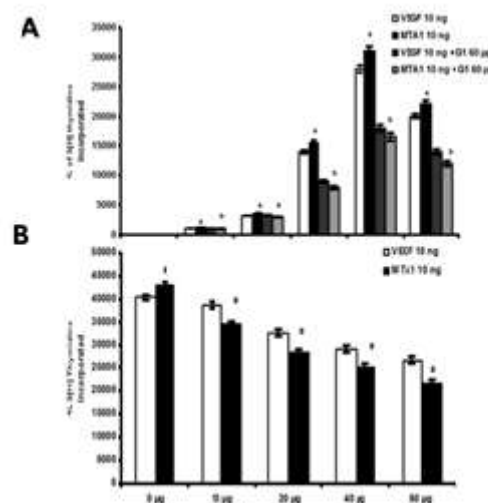


**Fig. 3** Induction of Blood Vessel in shell less Chorio Allantoic Membrane (CAM) by VEGF or MTA1 is inhibited by F6 (G1). Chick embryos were cracked out of their shells on to clingfilm hammocks. Egg preparations was covered with sterile petridish and transferred back to incubator. On day 5 a sterile filter disc (2 mm) with PBS or VEGF (10 ng/ml) or MTA1 (10 ng/ml) or F6 (G1) (60 µg/ml) were administrated on the CAM.

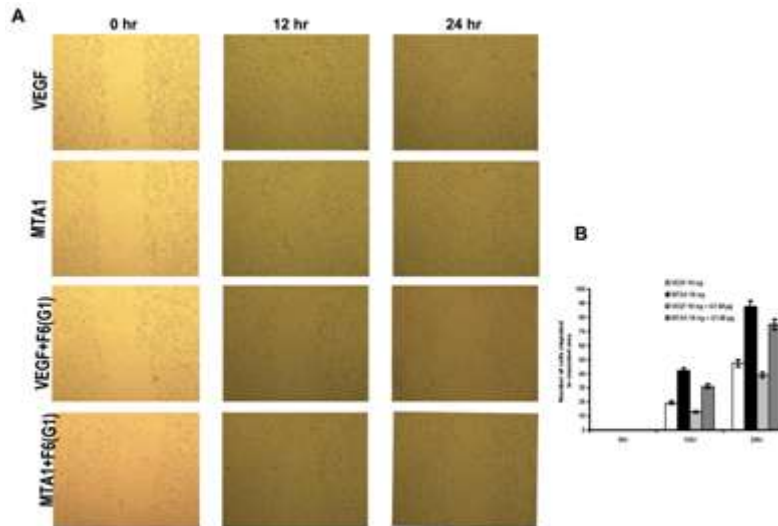


**Fig. 4** Induction of endothelial cell tube formation by VEGF or MTA1 by inhibition of F6 (G1).

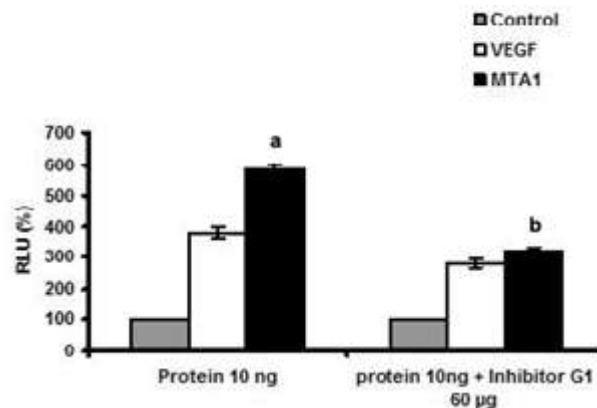
**A** Tube Formation Assay in Endothelial cells. Formation of tubes in HUVECs using recombinant MTA1 and VEGF proteins. As described in detail in “materials and methods”, HUVECs ( $5 \times 10^3$  cells per well) were seeded onto Matrigel along with MTA1 or VEGF proteins (10 ng/ml) or F6 (G1) (60 µg/ml) respectively. After incubation for 16 h at 37°C, capillary network were photographed and quantified using CCD camera at 40X magnification. **B** Quantification of tubes formed in recombinant MTA1 or VEGF or F6 (G1) in HUVECs. NIH image J was used to determine the total length of tube-like structures in images captured. The data shown is the mean  $\pm$  SD of three independent experiments. a= statistically significant at  $P < 0.05$  when MTA1 compared with VEGF and b= statistically significant at  $P < 0.05$  when MTA1+F6 (G1) compared with VEGF+F6(G1).



**Fig. 5** Effect of F6 (G1) on VEGF or MTA1 induced proliferation of MDA-MB-231 cells. (A, B) As described in detail in materials and methods, MDA-MB-231 ( $1 \times 10^5$  cells per well) were cultured *in vitro* in a six-well plate and processed for proliferative activity of recombinant VEGF or MTA1 (10 ng/ml) with inhibitor F6 (G1) in a time dependent manner (0, 6, 12, 24, 48 & 72 h) respectively. And F6 (G1) in dose-dependent manner (0, 10, 20, 40, 60  $\mu$ g/ml) respectively, using  $^3$ [H] Thymidine (1  $\mu$ Ci/ml of medium). The data shown is the mean  $\pm$  SD of three independent experiments. a= statistically significant at  $P < 0.05$  when MTA1 compared with VEGF and b= statistically significant at  $P < 0.05$  when MTA1+F6(G1) compared with VEGF+F6(G1).



**Fig.6** Effect of F6 (G1) on VEGF or MTA1 on closure of wounded area in MDA-MB-231.(A) As described in detail in materials and methods, MAD-MB-231 ( $2 \times 10^5$  cells per well) were seeded in a six-well plate and cultured in DMEM medium. The cells were serum starved overnight and a scratch was made on the cell monolayer. Cell debris was washed and the cells were cultured in medium containing with or without VEGF or MTA1 (10 ng/ml) or F6 (G1) (60  $\mu$ g/ml). The wound closure was photographed at different time intervals (0, 12 & 24 h). B. Quantification of the cells involved in wound closure. The cells that moved in the wounded area was counted and expressed as movement of control.



**Fig. 7** Effect of F6 (G1) on VEGF or MTA1 induced VEGF gene expression. As described in detail in “materials and methods”, MDA-MB-231 ( $2 \times 10^5$  cells per well) was seeded in six-well plates and transiently transfected with 2  $\mu$ g of VEGF promoter-luciferase reporter construct. Cells were treated either with VEGF (10 ng/ml) or MTA1 (10 ng/ml) or F6 (G1) (60  $\mu$ g/ml). Forty-eight hours later, cells were assayed for luciferase activity (luc), and  $\beta$ -Galactosidase ( $\beta$ -Gal) activity was used to normalize as internal control. The data shown is the mean  $\pm$  SD of three independent experiments. a= statistically significant at  $P < 0.05$  when MTA1 compared with VEGF and b= statistically significant at  $P < 0.05$  when MTA1+F6(G1) compared with VEGF+F6(G1).